

Case Report
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Acetyl-L-Carnitine and Nicotinamide for Prevention of Type 1 Diabetes. I-Literature Review which Gave Support to the Treatment. II-Case Report, Evaluation of Five Years Treatment

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Abstract

In the first part, this article review the accepted knowledge of type 1 diabetes, its physiopathology, the importance of cytokines and the induction of apoptosis and necrosis during its evolution. Throughout this work we describe in more detail the inhibition of this mechanism of cell destruction by acetyl-L-carnitine and nicotinamide. We also explain the complementary action of their association which gave support to the treatment.

In the second part, we present the complete evolution of 8 children treated with the oral medication of 50 mg/Kg of acetyl-L-carnitine plus 25 mg/Kg of nicotinamide during 5 years. We published the first 2 years of evolution under treatment in these children (JPEM 26: 347, 2013). The children had positive auto-antibodies and were consanguineous of type 1 diabetic patients. The intravenous glucose tolerance test (IVGTT) showed a first phase of insulin release minor of 48 μ U to enter in the protocol, and the same test was used for children evolution. Seven out eight children stopped the treatment because they normalized the metabolic parameters and no one became diabetic. All children increased the insulin response to IVGTT (between 1.44 to 5.69 times). Along the treatment, seven of these eight children turned their positive auto-antibodies into negatives.

Keywords: Autoimmune type 1 diabetes; Apoptosis and necrosis; Acetyl-L-carnitine plus Nicotinamide; Insulin increments; Case report

Introduction

The number of diabetic patients was estimated in 366 million people by a recent report of the World Health Organization, and this population would be doubled by the year 2030, (<http://www.idf.org/media-events/press-releases/2011/diabetes-atlas-5th-edition>) reaching 552 million people and the estimated diabetes increment is 3% per year, with fluctuations among different countries. From all these patients, insulin-dependent diabetes represents 7-10% of established clinical diabetes, and although type 1 diabetes is predominant in the infancy and adolescence, it can be present at every age. For further information about the presentation, evolution and prevention of type 1 Diabetes the interested reader, there are many excellent reviews [1-4].

Trough revision of literature we will present a brief comment about the current concept on the physiopathology of type 1 diabetes, and further detailed information on the actions of acetyl-L-carnitine and nicotinamide about the inhibition of pro-inflammatory cytokines, apoptosis and necrosis of pancreatic β -cell. Then we will present the results in a group of children treated during 5 years with acetyl-L-carnitine plus nicotinamide. It is worth highlighting that a previous communication after short period of treatment has been published [5].

First Part - Literature Review Which Gave Support to the Treatment
Current concepts on type 1 diabetes

It is accepted that before the appearance of clinical type 1 diabetes there is a period with a progressive decrease of β -cell mass with a reduction of insulin that is available. Finally, this fall of insulin became an insulin deficit and the patient evolved to insulin dependent diabetes.

This evolution was related to the genetic predispositions (HLA haplotypes DQB1 *0201, *0302, DRB1 *0301) and in populations with more frequents haplotypes DQA1 *0501-DQB1 *0201 and DQA1 *0301-DQB1 *0302 [4]. This genetic predisposition could be associated to other factors, such as viral infections (measles, mumps, among others) to initiate the autoantibody generation and the beginning of auto-aggression to pancreatic β -cell. The autoantibody found were about islets cells (ICA), glutamic acid decarboxylase o GAD65 antibodies (GADA), proinsulin/insulin (PAA/IAA), tyrosine phosphatase-like (IA2A), and zinc transporter-8 (ZnT8) [6,7]. The mechanisms involved in β -cell destruction are not explained by these known auto-antigens without included macrophages, dendritic cells, T and B lymphocytes. The sequences that finally trigger the immune-aggression could be described; 1) in response to antigen presentation (APCs) in the local pancreatic lymph node CD4⁺T lymphocytes proliferate and differentiate into auto-reactive CD4⁺T cells (effectors T cells, Teffs); 2) the activated Teffs release cytokines included IFN- γ and IL-2 which promotes the recruitment of cytotoxic macrophages CD8⁺T lymphocytes that release granzyme/perforin and increment CD8⁺ toxicity on β -cells. These

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lymphocytes release into the pancreatic islets the pro-inflammatory cytokines IFN- γ , TNF- α , IL-1 β and accumulate more inflammatory CD8⁺T cells (insulinitis). This process promotes further mononuclear cells recruitment [8].

There are animal models for type 1 diabetes, like C57BL/6J inbred mice and non-obese diabetic (NOD) mice. C57BL/6J develops an autoimmune response when treated with multiple sub-doses streptozotocin and evolves to type 1 diabetes with mononuclear accumulation (insulinitis) in pancreatic islets. The autoimmune response in C57BL/6J inbred mice has been demonstrated by numerous authors, and the immune-aggression was measured [9-12]. NOD mice are a model of spontaneous type 1 diabetes with autoimmunity against β -cell with islets insulinitis [13, 14] which allowed researches to study autoimmune evolution that evolves to type 1 diabetes. It is worth mentioning that young NOD mice were protected from spontaneous diabetes when transplanted with NOD islets from NOD mice treated with streptozotocin [15].

Current knowledge showed the mitochondria as the place for development of cellular apoptosis. However, as described, different stimuli in the cellular membrane can activate the acid sphingomyelinase (Sm) producing ceramide. It was known that some cytokines were important in ceramide production like tumor necrosis factor alpha (TNF- α), interleukine-1 beta (IL-1 β) and interferon-gamma (IFN- γ) [16]. Notwithstanding, last time, it has been suggested that ceramide appears later in the evolution of apoptosis, decreasing its role in the apoptosis development [17-21]. Another point worth mentioning was the caspases activation in cytoplasm by "dead receptors", TNF- α , irradiation, drugs like daunorubicin, staurosporin or other factors [22,23].

In mitochondria, the level of peroxidation (with ROS production), nuclear DNA damage due to radiation or some alteration in the electron transport chain can induce the cytochrome-c release from inner mitochondrial membrane with detention in electron transport and changes in the relation ATP/ADP which is an apoptosis signal [11,17]. This evolve sequentially in the association of protein Bax to porin (Vdac), changing the inner membrane permeability with the release of Bcl-2 [17] facilitating the cytochrome c release from inner membrane. With the alteration of electron transport chain and the permeability of inner mitochondrial membrane, the membrane potential and the oxidative phosphorylation were lost, a signal of mitochondrial damage and apoptosis [11,17]. The following step in the apoptosis sequence can be the formation of cytoplasm apoptosome with caspases activation [19,24] and ceramide production. It is worth highlighting that this evolution can increase peroxidation and mitochondrial damage [24].

Cytokines participation in this chain of reactions include IL-2 and IFN- γ after T cells activation and the rupture of lymphocytes equilibrium (Tregs/Teffs), resulting in recruitment of cytotoxic macrophages and CD8⁺T lymphocytes. In autoimmune diabetes, there is a cellular response of auto-aggression with nitric oxide production (NO) which is pro-apoptotic due to ROS induction [10,17,19,25]. The intracellular NO increments, higher than physiologic, can induce apoptosis. The extra-cellular augments of NO increase Fas expression in the cellular membrane, stimulate TNF- α and induce damage in nuclear DNA [19].

There is equilibrium between the regulatory T cells (Treg) and Teff cells which maintain peripheral tolerance, and its rupture not only predisposes to type 1 diabetes but other autoimmune illness [8]. The insulinitis with macrophages accumulation produce destruction of β -cell

more frequently by apoptosis than necrosis, as described by Jae-Hyoung et al. and Saisho et al. [26,27]. Depending the class of macrophages stimulation, Th1-type induce cell-derived IFN- γ and proinflammatory cytokines like TNF- α , IL-12, IL-1 β , IL-23 and toxic mediators like ROS and NO, with the expression of inducible nitric oxide synthase. These macrophages have increased antigen presentation ability [28]. The factor/s which triggers the immune-aggression and finish in cellular apoptosis and necrosis of β -cell are not completely know, but this knowledge would be required for immune-modulation [8,9].

Interesting enough, the cellular inductions of nitric oxide synthase can increment the mitochondrial NO and produce apoptosis by activation of pro-apoptotic Bax protein [19]. The immune response induced apoptosis and necrosis by activation of interleukin-1 β converting enzyme and cysteine protein protease-32-like proteases (homologous to gen *Caenorabditis elegans* Ced 3) [19,29] and activating by cleavage the poly-(ADP-ribose) polimerase (PARP) [29].

All this information enables us to propose whether in normal conditions there is equilibrium between β -cell destruction (apoptosis) and regeneration in the autoimmune process the regeneration is scarce due to an increment in cellular deaths which does not give time for β -cell regeneration. The process is out of equilibrium and directed to cellular destruction. Although the way is to stop the autoimmune process, there is a possibility of modifying this evolution by decreasing apoptosis and necrosis and increasing β -cell regeneration.

Inhibition of apoptosis and necrosis with acetyl-L-carnitine and nicotinamide

The possibility of inhibiting "in vivo" and "in vitro" the apoptosis and necrosis with the administration of L-carnitine [30-32], acetyl-L-carnitine [33] and nicotinamide [34,35] is well known. It was proposed that the degree of inhibition of carnitine palmitoyltransferase-1 (CPT1) should be a mitochondrial mechanism of cellular apoptosis, but current studies suggest that apoptosis was more dependent on fatty acid transported than the inhibition level of CPT1. The palmitoyl-carnitine contributed to apoptosis when it was in evolution, but in its absence the palmitoil-carnitine did not induce it [36]. The palmitate inhibited CPT1, induced ceramide accumulation, inhibited the mitochondrial electron transport and induced cytochrome-c release [36]. A possible explanation about the protection of apoptosis by acyl-carnitines, opposites of apoptosis increments by palmitoyl-carnitine were supported that palmitate, and specifically the palmitoyl-CoA, competing with mitochondrial acetyl-transferases and increments mitochondrial palmitate [37]. Otherwise palmitoil-CoA was the limiting step in ceramide synthesis and its increase could contribute to an increment of ceramide [38].

The acyl-carnitines activated CPT1 [37] and L-carnitine or acyl-carnitines protected the development of apoptosis induced by "dead receptors" like CD95 (also known as Fas or Apo1) while palmitoyl-carnitine increased apoptosis [38]. L-carnitine at doses of 200 mg/Kg/48 h increased "in vivo" the anti-apoptotic hormone IGF-I restoring its levels decreased in diabetes by streptozotocin [39]. NOD mouse model was protected when transferred with autoreactive T cells treated with IGF-I. The hormone plays an integral role in the development and functions of β -cells because IGF-I stimulates β -cells growth. The hormone can modulate the immune response in diabetes because it expressed and maturated CD3⁺T and CD8⁺T cells in thymocytes and splenocytes [40]. Thus, L-carnitine through the increment of IGF-I can modulate the immune response.

L-carnitine interacted with cardiolipin in the inner mitochondrial membrane, stabilizing the membrane and protecting the mitochondrial function [41]. L-carnitine in mitochondrial matrix is the transporter of metabolic residues (acetylcarnitine, propionylcarnitine, malonylcarnitine, among others) as well as xenobiotics, acting for mitochondrial detoxification [41]. The antiapoptotic activity of L-carnitine was demonstrated through its prevention in ceramide production and the regulation of caspases activities (caspase 8 as the beginning of apoptosis and caspase 9 in the chain of activation) [24].

In isolated hepatocytes, the addition of L-carnitine did not increase the β -oxidation but incremented the availability of mitochondrial acylcarnitines [38] suggesting that L-carnitine in normal concentration was sufficient for high β -oxidation activity. It has also been observed that peroxisomal acylcarnitines can cross to mitochondria by diffusion without CPT1 activation [42] although the metabolic implication of this transport was not quantified.

Moreover, it was demonstrated in salmon that high and constant doses of L-carnitine decreased the fat content and increased proteins in tissues [43]. In this experimental model, the were observed with L-carnitine: an increased amino acid synthesis from the Krebs cycle with high metabolic flux through pyruvate carboxylase (PC), incremented oxaloacetate and decreased branched-chain amino acid degradation. Under normal conditions, increments of citrate increased oxaloacetate with activation of acetyl-CoA carboxylase (ACC), which augmented malonyl-CoA with the secondary inhibition of CPT1. This evolution, in turn, inhibited mitochondrial transport of fatty acid with detention of β -oxidation. However, this chain of reactions did not occur in the experimental model with high doses of L-carnitine. An increment of 73% was observed in palmitate β -oxidation and 81% of the flux was increased through PC to produce oxaloacetate (due to an incremented synthesis of the enzyme). The branched-chain amino acids were incremented most likely due to a decrease in the activity of mitochondrial enzyme branched-chain α -ketoacid dehydrogenase complex (BCKDC). There was an augmented amino acid synthesis (between 7% and 112% depending on amino acid considered), incremented oxaloacetate which increased gluconeogenesis between 120% and 210%, and an incremented protein synthesis between 20% and 60% due to pool availability of essential and non-essential amino acids [43]. The feedback inhibition due to increments in citrate and malonyl-CoA was not observed because:

- 1- The acetyl-CoA did not increase because the production was in equilibrium with the consumption.
- 2- The ATP/ADP relationship was unchanged because the acetyl-CoA consumption was directed to protein synthesis.
- 3- There were neither citrate nor acetyl-CoA increments because both were in equilibrium. Then, there was not an increment of ACC activity.
- 4- There was no detention of β -oxidation because there was not malonyl-CoA increment [43].

The metabolic activity was similar to the “knock out” model of mitochondrial isoenzyme of acetyl-CoA carboxylase [44]. Consequently, it can be postulated that increased doses of L-carnitine produce more energy by fatty acid oxidation and increased anabolism due to pyruvate availability. Insulin and palmitoyl-carnitine inhibited the BCKD complex and augmented the branched-chain amino acids at disposition [45,46]. The treated salmon showed high protein content with less grass storage than the untreated salmon, with a significantly

higher amount of branched chain amino acids in tissues and blood [43]. The interpretation of this behavior was an increased glycolysis by insulin with more available ADP for ATP synthesis (as explained by hexokinase shunt) [47] and pyruvate in mitochondrial matrix, with an incremented Krebs cycle activity and oxaloacetate availability.

Acetyl-L-carnitine has a similar anti-apoptotic activity than L-carnitine. The same as L-carnitine incremented the proteins synthesis and decreased grass storage by increased fatty acid β -oxidation with ATP increments. Moreover, the leptin resistance was reduced in old animals [33], and also the apoptosis was reduced as shown in cultures of “Jurkat cells” [37] and neuronal cells [48]. The induction of ATP synthesis was fast because acetyl-L-carnitine came directly into mitochondria, participated in the pool of acetyl-CoA and the acetyl group was consumed in the TCA cycle [49]. It has been demonstrated that L-carnitine and acetyl-L-carnitine in normal subjects augmented the glucose storage in tissues, with less glycogenolysis, increasing insulin sensitivity and glucose oxidation [50-52]. Likewise, many experimental works showed the protection of acetyl-L-carnitine on nerve system [53,54] and protein glycation in diabetes [55]. It was published that treatment with L-carnitine increased fatty acid transport but no β -oxidation [30]. New studies showed that increments of L-carnitine augmented β -oxidation of saturated and non-saturated fatty acids [56,57].

Acetyl-L-carnitine treatment induce the activity of coactivators PGC1- α /PGC1- β of peroxisome proliferator-activated receptor gamma which participates in mitochondrial biogenesis and signaling pathway, promoting in old liver rats a new mitochondrial population [58]. L-carnitine diminished in tissues with the age, especially in males, but in blood it remained normal [59]. In diabetes L-carnitine was found consistently decreased in blood [57-59]. It was suggested that L-carnitine was lost by urine [60]. Also, it has been proposed that it is convenient to treat diabetic patients with L-carnitine [61,62].

It has been reported by La Marca et al. [63] low concentrations of L-carnitine and its derivatives in neonates at birth and the authors showed the potential of L-carnitine assay in diagnostic of type 1 diabetes. They also proposed the treatment with supplementary L-carnitine to improve the thymic tissue-specific auto-antigens, increasing thymic “central tolerance” avoiding auto-aggression.

Following the above physiological descriptions, it can be postulated that pharmacological increments of L-carnitine or acetyl-L-carnitine inhibits cell apoptosis and increase fatty acid oxidation and tricarboxylic acid cycle activity.

Another mechanism of apoptosis inhibition was nicotinamide, a precursor of NAD⁺ [64]. Nicotinamide impeded the activation of caspases 3 and 9 maintaining the stability of Vdac in vascular endothelium [34]. However not only has it done it, also protect the mitochondrial membrane potential and impede the cytochrome c release, preventing the induction of caspases 8, 1 and 3 [65]. Large doses of nicotinamide also prevented the diabetic evolution in NOD mice and decreased the pancreatic insulinitis [66,67]. The same protective effect was observed with isonicotinamide (a nicotinamide derivate) in mice became diabetic after receiving 3 different doses of streptozotocin [68]. In primary cultures of isolated hepatocytes from adult normal rats, 10 mM of nicotinamide associated to insulin and epidermal growth factor prolonged the cultures for more than one month [69].

Nicotinamide inhibited the gene of poly (ADP-ribose) polymerase (PARP) a generator of cellular necrosis [35]. Its activation was produced by the proteolytic cleavage of cysteine-protein 32, simil protease (CPP)

named apoptain. The proteolytic activation of PARP was also produced by interleukin-1 β simile-converting enzyme, and this enzyme (IL-1 β) has been described as the apoptosis initiator in type 1 diabetes [13]. PARP rebuilt damaged DNA and this function required normal concentrations of NAD⁺. When NAD⁺ was not sufficient for its function, the enzyme was activated in non-reversible form and consuming ATP until cellular necrosis occurred [35]. Nicotinamide was a potent inhibitor of pro-inflammatory cytokines through inhibition of PARP [70,71], and this activity was not only based on its anti-apoptotic and anti-necrotic action due to PARP gene inhibition but also on its capacity to be a NAD⁺ substrate [64,67]. With an enzyme Km for DNA of 86.5 μ M, and an apparent Km for NAD⁺ of 20 μ M [70, 72-74], a nicotinamide dose in humans of 25 mg/kg body weight reached a plasma concentration of 0.3 mM [64] demonstrating that nicotinamide at this dose was sufficient for PARP inhibition [70].

Objectives to reach in acetyl-L-carnitine plus nicotinamide administration

In cellular response of auto-aggression with high NO production and secondary ROS induction, as demonstrated in autoimmune diabetes [10,17], acetyl-L-carnitine and nicotinamide could act by decreasing the apoptotic response and by giving time to cellular regeneration. This reasoning could be applied to autoimmune prediabetes demonstrated by auto-antibodies, a decreased first phase of insulin release, and low integrated insulin area after glucose stimulation with high glucose values during intravenous glucose tolerance tests. We could hypothesize that high doses of acetyl-L-carnitine and nicotinamide could produce an increment in cytosolic NAD⁺ with activation of citrate-malate shuttle and a secondary increase in mitochondrial NADH, activating citric acid formation and TCA flux. Specifically, an increase in succinate or succinic acid monomethyl ester stimulated proinsulin biosynthesis in β -cells. The increment in energy, insulin, IGF-I, amino acid synthesis and the availability of branched chain amino acid are basic conditions for β -cells regeneration [75-77].

It is important to emphasize the absence of toxicity for these natural compounds [70,71,78,79]. The proposed doses of nicotinamide was demonstrated as without toxicity [70,71] and oral administration of very high doses of L-carnitine only produce diarrhea for osmotic reasons, without toxic signals [38]. However, the deficit of L-carnitine produced cellular damage with neurological manifestation [80] because L-carnitine and its derivatives are physiological regulators of peroxisomes interaction with mitochondria [57]. Its administration was recommended during infancy and old age [70,81].

Studies supporting the proposed treatment

A number of studies demonstrated that cytokines could be modulated with the addition of acetyl-L-carnitine and L-carnitine [62]. We investigated if L-carnitine could modify the "in vitro" inhibition of insulin release of normal β -cells when they were co-cultured with splenocytes from diabetic mice [81]. We observed (Figure 1) that "diabetic splenocytes" decreased the insulin secretion from normal β -cells stimulated with 16.5 mmol/L of glucose plus 5.5 mmol/L of theophylline; and 200 μ g/well of L-carnitine can overcome this inhibition. The inhibition was probably due to cytokines released [82-84].

This study allowed us to think that the association of acetyl-L-carnitine and nicotinamide could have similar behavior in experimental animals. The combined treatment was studied in male C57BL/6J inbred mice, after inducing autoimmune diabetes with the injection of multiples low doses of streptozotocin. A prolonged treatment with 50

DIABETIC SPLENCYTES AND L-CARNITINE "In vitro" Co-culture

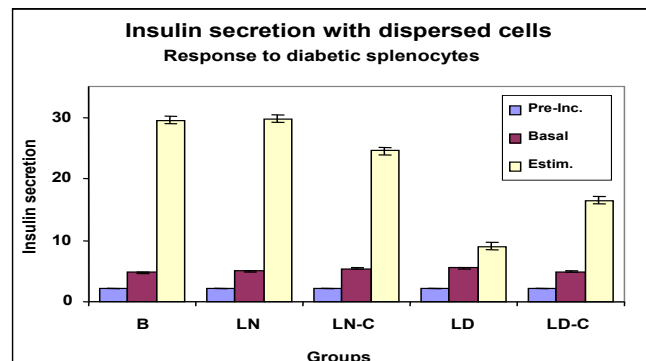


Figure 1: The islets cell suspension (100 μ l/well) was 5×10^3 cells. The incubation was 18 hours at 37°C, alone or with addition of 4×10^6 mononuclear cells from "control" or "diabetic" mice. The limit of insulin secretion under stimulation of 16.5 mmol/L glucose plus 5.5 mmol/L theophylline was more than 25 μ U insulin/5000 dispersed islets cells in 5 min. Below this value it was assumed as "immune aggression" [82].

B: basal insulin secretion; **LN:** insulin secretion with normal splenocytes; **LN-C:** insulin secretion with normal splenocytes and 200 μ g/well of L-carnitine; **LD:** insulin secretion with "diabetic" splenocytes; **LD-C:** insulin secretion with "diabetic" splenocytes and 200 μ g/well of L-carnitine. The bars show Mean \pm SEM (n: 5 replicates).

Statistic

B: 29.57 ± 0.33 ; **LN:** 30.06 ± 0.38 ; **LN-C:** 24.57 ± 0.38 ; **LD:** 9.44 ± 0.43 ; **LD-C:** 17.04 ± 0.40 . **B vs. LN:** no sig.; **B vs. LN-C:** $p < 0.001$; **B vs. LD:** $p < 0.0001$; **B vs. LD-C:** $p < 0.0001$; **LN-C vs. LD-C:** $p < 0.002$. L-carnitine decreased the insulin secretion of isolated islet cells but overcame the inhibition of "diabetic" splenocytes.

mg/Kg of acetyl-L-carnitine and 25 mg/Kg of nicotinamide resolved the immune diabetes induced in mice [82]. The associated treatment normalized the glucose levels, plasma insulin and normalized the β -cell mass. After 30 days of treatment, a negative evolution was observed in all parameters under study, and after that, it was observed that all the same parameters were improved (Figure 2). There was a first peak recovery observed in perfused pancreas and the β -cells mass of islets was normalized with normal insulin content. The immune aggression evolved from 9 to 20 (normal >25).

Another publication with the association of acetyl-L-carnitine, nicotinamide, lipoic acid and biotin improved mitochondrial biogenesis in type 2 diabetic Goto-Kakizaki rats. The treatment increased complex I and II in mitochondrial respiratory chain and the genes involved in lipid metabolism [85].

It has been recently published that in rats fed with a sucrose rich diet (which produce insulin resistance) a treatment with 50 mg/Kg of acetyl-L-carnitine and 25 mg/Kg of nicotinamide reduced the insulin resistance, increased the insulin secretion and normalized the blood glucose; thus increasing the glucose disappearance rate (Kg). The treatment diminished triacylglycerol in muscle and plasma, decreased hepatic steatosis, and normalized CPT1, improving ACC and fatty acid synthase [86].

Second Part - Case Report, Evaluation of Five-Years Treatment

Epidemiologic studies showed that siblings with type 1 diabetes had 6-10% of potential risk for this illness, but it was observed that only 10-15% of children with this potential risk developed clinical diabetes [1].

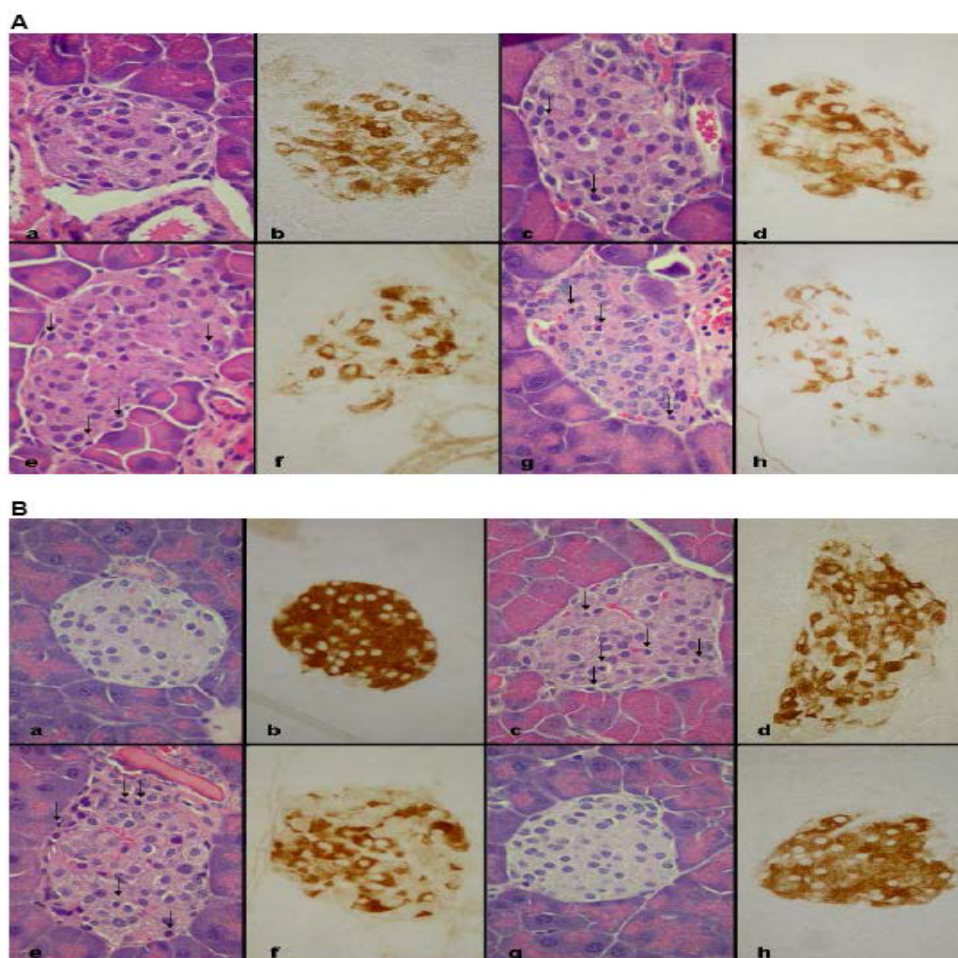


Figure 2: Technique: hematoxylin-eosin and insulin immunostained (400X)

A - Diabetic group (not treated): **a-b** day 8; **c-d** day 12; **e-f** day 30; **g-h** day 110.

B - Control group (normal islet): **a-b**. Diabetic group (treated): **c-d** day 12; **e-f** day 30; **g-h** day 110. The arrows showed mononuclear cells.

The islets recover the insulin stained mass and its normal size after 110 days of intraperitoneal injection of 50 mg/Kg of acetyl-L-carnitine and 25 mg/Kg of nicotinamide.

This potential risk was increased if the siblings had the genetic predisposition (specially the alleles DQB1*0302/*0201 of high susceptibility) which increased the possibility of developing auto-antibodies. The presence of more than one autoantibody increased the statistical value of developing type 1 diabetes [1,3]. Finally, preclinical diabetes was suspected when the immunologic markers and susceptible genotypes were associated to metabolic parameters showing decreased insulin and abnormality in its secretory pattern, with an elevation of blood glucose during an IVGTT. Under these conditions it was estimated that 25-50% of these children evolved to type 1 diabetes in 5 years (DPT1 study, Diabetes Care 31: 146-150, 2008).

Material and Methods

We studied 70 siblings of type 1 diabetes patients treated with insulin, but only 64 were restudied. From them, 2 children developed clinical diabetes before a new study to confirm their inclusion in the program. This evolution showed, during the short period of observation, that children studied were of high risk of diabetes. We selected 9 children to be included in the treatment, and one of them developed clinical diabetes after 10 months of treatment. The other 8 children are present in this paper after 5 years of treatment.

The selections of these children were described in a previous work [5]. Briefly, all children were consanguineous of type 1 diabetic patients, and were positive for one or more auto-antibodies. GADA, PAA/IAA and IA2A were determined by reference radioligand binding assay (RBA). GADA results were calculated as GADA antibody index as previously described [87] and expressed as SD score. IA-2A was determined by RBA essentially as described for GADA using ICA512bdc gene, coding for residues 256-979 of IA-2A. PAA/IAA was determined by RBA and results were calculated as B% and expressed as SD score. All autoantibody assays were considered positive if SD score > 3 [87].

Polymorphism in HLA-DQB1 gene was typed using polymerase chain reaction (PCR) and sequence-specific oligonucleotide (SSO) probes with Kit Inno-LIPA HLA-DQB1 (Innogenetics, Zwijnaarde, Belgium) [88].

The children were studied with IVGTT and by means of the same test the following data was measured: the first phase of insulin response (FPIR < 48 μ U insulin) [89,90], the integrated area of insulin secretion, the glucose disappearance rates (Kg), the equation and HOMA-IR [91].

The value accepted for the integrated area of insulin was < 200 μ U

without peaks, but this condition is extreme and it was found in type 1 diabetic children of recent diagnosed [92].

The equation “ $\log n(\text{ins. area}) \times \text{Kg}$ ” (Eq) related the amount of insulin secreted under glucose stimulation to the glucose disappearance rate (Figure 3). It was interpreted as the functional expression of glucose response to the amount of insulin secreted in the IVGTT and it was adopted due to its simplicity and the requirement of few insulin samples.

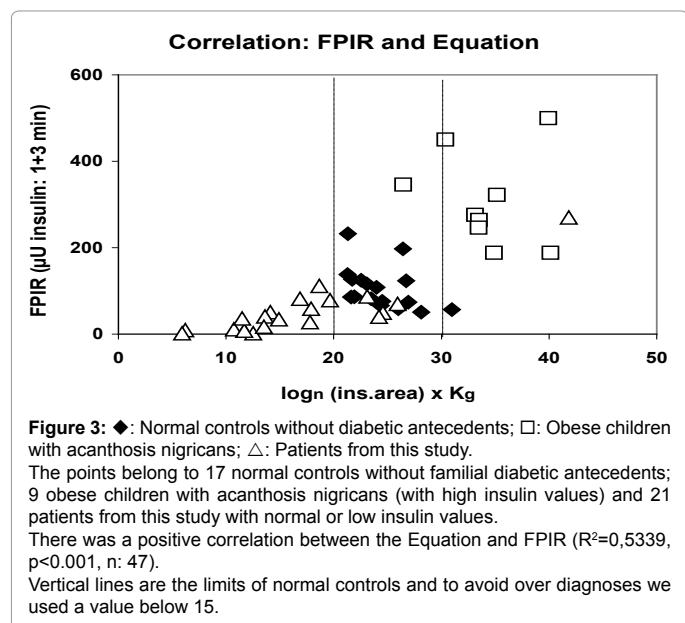
The normal pediatric glucose values for ISPAD (International Society for Pediatric and Adolescent Diabetes) during fasting were 100 mg%, intolerance between 100 mg% and 125 mg%, and over this value clinical diabetes was considered. The glucose values for IVGTT were 144 mg% (8 mmol/L) at 60 min (Joslin Diabetes Center). We found during an OGTT in 100 normal children a value of 126 mg% (+2 SD) at 120 min [93]. For these reasons we believe that glucose values higher than 126 mg% in IVGTT should be treated because it had been considered that a normal IVGTT at 60 min should have the same value or lower than the basal glucose value.

The statistical model was the “Intention to treat” (ITT) [94,95] which enables the researcher, when the child surpasses certain parameters previously described to change the group. The use of “Control” for children is not ethical, more when the treatment poses no risks. The parameters were: basal glucose > 110 mg%, glucose at 60 min > 126 mg%, $\text{Kg} < 1.41$ and $\text{Eq} < 15$.

The children started when they were recruited, and therefore the times of treatment differed. The conditions for recruitment took a long time and we decided to continue with the selected children at that moment. Throughout the study and due to the evolution, all children were treated.

Results

Two of these children showed a genetic predisposition and were homozygous for DQB1 alleles, and 6 were heterozygous. The most important results of previous evolution from the eight children are shown in black and the follow up is present in blue (Table 1). It can be observed that no one became diabetic during this prolonged evolution.



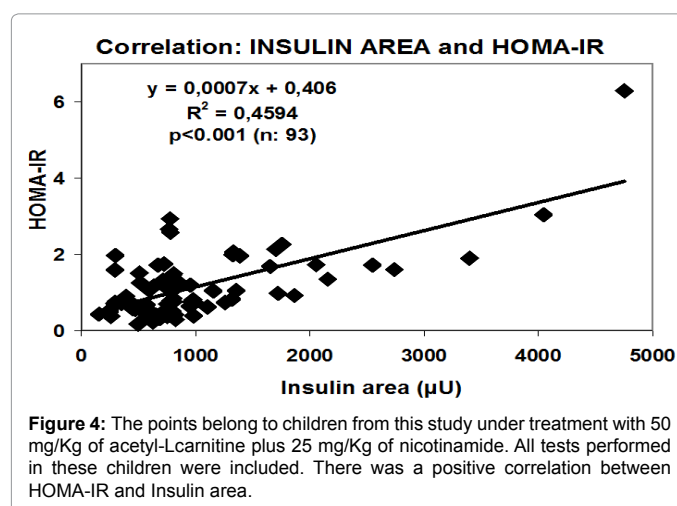
The children treated over a period longer than 1 year had a normal evolution in their metabolic parameters and they increased the total insulin under glucose stimulation (Table 2).

Out of 8 children 7 stopped the treatment and two among all patient required special attention and their evolution is described. The first studies of child RU were conducted in March 2008. He was randomly included in the group of non-treated patients, until October 2008 hyperglycemia was added to low FPIR. During his evolution he presented an integrated insulin area < 200 μU with glycosuria but clinically asymptomatic and normal growth rate. He was carefully followed without interrupting the treatment and finally he recovered his altered metabolic parameters. He had been on medication 5 years and 6 month (Table 2). He received half of the initial doses until a new study (June 2014) when we decided he stop the treatment.

The child PM started his treatment with a glucose value of 205 mg% at 60 min which was compatible with established clinical diabetes, but upon physicians' and parents' request, he was included in the protocol (with a prior clarification that he later receives the treatment). He persisted with low FPIR, low Eq. values and hyperglycemia but clinically asymptomatic with normal growth and education. The integrated insulin area increased with the treatment but the other parameters were kept without correction (FPIR, glucose values and HbA1c). During their studies this child normalized the HbA1c but with high HOMA-IR (Table 1) and we prescribed half of the doses of acetyl-L-carnitine and nicotinamide. In June 2014, in a new study, he normalized the glucose and HOMA-IR but the integrated insulin area, Kg and Eq. decreased in excess; for that reason we started again with the complete doses of acetyl-L-carnitine and nicotinamide.

The children PM and AF showed a fall of Kg and an increment of HOMA-IR after a rise of insulin. If we carefully observe all children during the treatment evolution, it could be established that insulin increments were followed by increments of HOMA-IR. The child PM is an example because he normalized his glucose values and HOMA-IR during a dramatic insulin fall, indicating that glucose is an important marker of insulin resistance and the treatment must be sustained still with high glucose values. When we plotted the total insulin area under glucose in IVGTT with the HOMA-IR, we found a significant correlation (Figure 4).

The insulin area in the IVGTT was incremented between 1.44 to 5.86 times (Table 2), and the insulin increments over 1000 μU were



Name	Date of study	Date of treatment	FPIR (μU)	"K _g "	Insulin area (μU)	HOMA-IR	Equation	Basal glucose (mg%)	60 min glucose (mg%)	Hb A1c
B.L. 4y 8m DQB1 *0201/0604 PAA: 0,36	2/28/00		30							
	2/16/01	8/6/01	88	1.34	526	0.63	8.4	64	57	
	8/4/03	"	31	2.88	774	2.93	19.16	88	79	
	2/8/11	"	36	1.71	823	0.29	11.48	68	94	
	6/14/11	"	61	3.34	1258	0.74	23.84	59	64	
	10/11/11	NT-8/14/11	80	2.23	2154	1.35	15.33	71	108	
	12/21/12		116	2.88	1865	0.92	21.69	71	129	5.5
O.M. 6y 5m DQB1 *0301/0601 PAA: Pos.	10/6/09	11/4/09	47	3.33	778	0.44	22.17	97	121	6.0
	5/17/11	"	--	4.27	430	0.74	25.89	112	114	
	10/25/11	NT-10/25/11	99	3.89	1324	1.99	27.96	99	101	
	6/12/12		123	3.81	1390	1.96	27.57	117	109	6.2
	6/4/13		81	3.90	1327	2.06	28.04	110	95	6.3
P.V.T.S. 8y 10m DQB1 *0302/0402 GAD: 0,75 IA2A: 0,04 PAA: 0,65	10/31/07	11/13/07	<2	0.92	611	0.40	5.90	56	169	
	3/17/08	"	41	2.18	474	0.56	13.19	82	74	
	1/5/09	"	38	4.86	749	0.56	32.17	76	72	7.0
	12/29/09	"	186	6.14	1650	1.68	45.49	80	56	
	9/21/10	NT-3/1/10	100	3.27	1756	2.26	24.43	90	111	5.1
	7/19/11		126	2.13	1699	2.13	15.84	74	82	5.4
	7/2/13					2.62		105		5.1
H.P. 17y 4m DQB1 *0302/0402 PAA: 8,17	4/26/07	4/26/07	9	0.88	1152	1,05	6.20	72	152	
	8/16/07	"	36	1.06	574	0,39	7.73	73	169	
	9/21/10	NT-9/1/09	49	2.07	1429	1,64	15.04	83	162	5.2
	7/16/13					1.11		88		4.8
A.N. 15 y DQB1 *0201/0201 GAD:0,01 PAA:1,50 IA2A:1,05	12/21/10	4/20/10	29	1.84	1354	1.05	13.27	73	73	5.4
	12/13/11	"	75	1.87	627	0.23	12.04	72	141	---
	5/24/12	"	51	2.39	1103	0.62	16.74	66	97	5.6
	8/24/12	"	51	1.93	977	0.81	13.28	85	---	5.9
	1/8/13	"	71	1.87	1315	0.82	13.43	67	125	6.3
	3/5/14	"				1.17		82	109	5.7
		NT-3/5/14								
A.F. 7 y DQB1 *0302/0201 PAA: 6,35	3/1/10	5/12/10	48	4.19	752	1.67	27.75	103	102	5.8
	5/10/11	"	108	2.48	1198	2.40	17.58	110	112	7.1
	9/20/11	"	77	3.02	776	1.87	20.09	119	111	7.1
	6/9/12	"	78	1.68	1086	2.33	11.74	98	125	6.4
	11/13/12	"	91	2.39	1504	2.28	17.48	112	121	5.9
	4/9/13	"	70	4.56	1023	2.22	31.60	113	111	7.9
	9/3/13	"	57	1.48	904	2.17	10.07	121	151	---
	5/21/14	"				3.15		117	139	6.1
	11/5/14	NT-11/5/14	125	2.48	1876	2.78	20.95	126	---	5.6
R.U. 5y 9m DQB1 *0402/0302 IA2A:1,41	5/12/09	10/16/08	<2	2.48	156	0.43	12.52	102	103	6.5
	4/26/11	"	51	3.84	637	1.17	24.79	108	106	8.3
	8/9/11	"	58	3.29	668	1.72	21.40	127	136	7.4
	10/18/11	"				1.20		125		7.0
	12/6/11	"	50	2.54	724	1.75	16.73	107	130	7.3
	5/15/12	"	80	2.10	807	1.49	14.06	156	170	6.7
	9/4/12	"	89	3.10	838	1.30	20.86	118	---	7.1
	1/15/13	"	72	2.74	790	1.33	18.28	120	153	7.7
	5/7/13	"	70	3.03	756	1.20	20.08	117	144	6.1
	6/2/14	"	121	3.60	887	1.21	24.43	87	128	
		NT-6/10/14								
P.M. 7y 5m DQB1 00302/0103 PAA: 0,65	7/12/11	11/4/09	17	2.43	259	0.37	13.50	80	146	5.9
	11/1/11	"	27	1.53	391	0.90	9.13	100	140	6.6
	6/24/12	"	14	2.66	596	1.06	16.99	108	154	6.5
	10/16/12	"	47	3.74	779	2.58	24.90	130	130	7.1
	3/05/13	"	34	1.60	516	1.26	9.99	108	161	7.2
	7/23/13	"	44	1.23	768	2.66	8.17	124	178	6.9
	5/3/14	"				3.12		132		6.0
	6/2/14	"	28	1.03	373	0.95	6.10	111	112	

Black numbers: data selected from previous study (JPEM 26: 347, 2013). *Blue numbers:* new studies. Abnormal values appear in bold type, and underlined the glucose values accepted as intolerance for ISPAD or Joslin Diabetes Center *HLA-DQB1. Antibody concentrations are in the first column in bold type.

*Positive glycosuria. **Half doses of treatment. NT: No Treatment.

Table 1: Individual evolution of children.

Children	Minor value (date)	Final value (date)	After suspension (date)	Insulin increments
BL	526 µU (8/01)	1258 µU (6/11)	1865 µU (12/12)	Final - 2.39 After Susp. - 3.55
OM	430 µU (1/09)	1390 µU (6/12)	1327 µU (6/13)	Final - 3.23 After Susp. - 3.09
HP	494 µU (1/08)	751 µU (8/09)	1429 µU (8/09)	Final - 1.52 After Susp. - 2.89
PVTS	474 µU (8/08)	1756 µU (1/10)	1699 µU (7/11)	Final - 3.70 After Susp. - 3.58
AN	627 µU (12/10)	1315 µU (1/13)	1315 µU (1/13)	Final - 2.10 After Susp. - 2.10
AF	424 µU (6/08)	1876 µU (11/14)	1876 µU (11/14)	Final - 4.42 After Susp. - 4.42
RU	156 µU (5/09)	756 µU (5/13)	887 µU (6/14)	Final - 4.85 After Susp. - 5.69
PM	244 µU (3/11)	373 µU (6/14)	-----	Final - 1.44

The evolution of integrated insulin area was determined with an IVGTT during the study. Dates were between parenthesis.

Minor value: the lowest value reached in the integrated insulin area during the study.

Final value: the last value obtained during the study.

Susp: the last integrated insulin value obtained after suspension.

Table 2: Integrated insulin area during ivgtt.

Children	Weight		Eight		Time
	Initial	Final	Initial	Final	Treatment
BL	25-50	50	50	75	2 years 10 months 4 y 4 m (half doses)
OM	75	90	97	97+	2 years 11 months
HP	50	50-75	75	75	1 year 10 months
PVTS	25	25-50	25	50	1 year 9 months 8 m (half doses)
AN	50	50	90	75	3 years 11 months
AF	75	90	97	97+	4 years 2 m (half doses)
RU	25	50	50	75	5 years 6 months 1 m (half doses)

We describe the clinical parameters, antibodies and time of evolution of children under study. Weight and height are expressed in percentiles. "Initial" was the first determination after to enter at the study, and "Final" was the last determination. y: Years; m: Months; NEG: Negative. The **P97+** signifies that these children changed their percentiles over 97. As described in the table, the time of treatment (total dose or half of dose).

Table 3: Evolution of children.

DATE OF ASSAY	GADA		PAA		IA-2A
April 2007	POS	0.05	NEG		NEG
September 2008	POS	0.044	NEG		POS
January 2009	POS	0.049	POS	4.607	NEG
July 2009	POS	0.052	NEG		NEG
December 2010	POS	0.03	NEG		NEG
August 2011	POS	0.075	NEG		
July 2013	POS	0.086	NEG		

Autoantibody evolution during treatment (bold numbers), and after suspension of treatment (from December 2010, normal numbers). The values are after subtraction of non-specific.

Table 4: Autoantibody in child pvts.

observed in 6 children out 8. Six of 8 children incremented their height and change their percentiles before pubertal development, included when they were over P97. The only two children without changes in their percentiles were treated between 16 and 17. All final percentile values were obtained in the last study 90 Clinically all children presented a good evolution, without intercurrents or intolerance to treatment. They were lean, tall, with good scholar learning, activity and with a better aspect (body skin and hair) in

comparison with their brothers whether they are diabetics or not (Table 3).

During the study we found that positive auto-antibodies became negatives and this finding was not foreseen in the protocol. Only a child (PVTS) maintains a positive GADA antibody which decreased during the treatment and increased when he stopped the treatment (Table 4).

Discussion

From the experimental studies in mice, we deduced that the time of treatment had to be prolonged. Moreover, the previous conditions to initiate the treatment require a sufficient mass of β -cells to be regenerated. This mean that children with metabolic parameters suggesting a very high insufficient β -cells mass could not start the treatment. This theoretical considerations lost relevance when they were contrasted with the results obtained with the treated children.

Many of them, if not all, would be type 1 diabetics and the recovery obtained was with the administration of 3 tablets a day. The results suggested that treatment sustained for more than 1 year should be continuous still if the metabolic parameters were without correction as observed in RU and PM. RU is an example of this concept because he was treated even with an integrated insulin area < 200 µU (tonic insulin secretion) glyated hemoglobin over 8 with glycosuria, and a prolonged treatment allowed him to recover metabolic parameters and suspend the treatment in June 2014. Naturally, the children need a continuous medical control.

Insulin resistance was observed after insulin increments, but this behavior was not a response to the proposed treatment it is a general response to insulin increments, not only by endogenous secretion but even during insulin treatment [89]. In these children, the protection to this behavior could be, after obtaining the recovery of insulin secretion, a reduction to half of the doses because in the cases in which it was done, the results were equally successful.

It was impossible to understand diabetes without taking into account the participation of mitochondria because succinate and oxaloacetate induce the synthesis of proinsulin. Nicotinamide is a NAD⁺ substrate for mitochondria [64,67]. Otherwise, NAD⁺ participates in regulation of BCKDC and also in TCA flux. When an increment of

NAD⁺ is required, because the chain of reactions in the TCA cycle is not sufficient, cytosolic NAD⁺ is transported to mitochondria through pyruvate/citrate shuttle. This requires an excess of mitochondrial acetyl-CoA that can be provided by acetyl-L-carnitine. This behavior was similar to hexokinase shunt to increase ADP when ATP synthesis was urgent required [47]. Acetyl-L-carnitine be metabolized rapidly when it was injected IV because it was transported to mitochondria directly and acetyl moiety was released into mitochondria by acetyl-transferase [38]. This acetyl-CoA was part of acetyl-CoA pool and it participated in the synthesis of glutamate, glutamine, GABA, etc. As metabolic substrate in the TCA cycle, the acetyl groups could increment succinate and oxalacetate, increasing the anaplerosis and amino acid synthesis. The L-carnitine can be transported outside mitochondria associated to other groups (propionyl, octanoyl, or the same acetyl, etc) and L-carnitine started again in other cycle [38].

Pharmacologic treatment with acetyl-L-carnitine and nicotinamide generated acetyl-CoA and increased mitochondrial NADH with incremented TCA flux [40,47,62]. The incremented energy required enzyme synthesis, but this demand is first energetic and secondarily enzymatic, giving time for enzyme synthesis. As described above, the treatment required mitochondrial substrates which were resolved by its own metabolic activity.

We observed during the study that children with specific positive antibodies became negatives. Our children did not show high antibodies titer and we thought that it was the reason for its disappearances. An explanation for this antibody evolution could be the induction and increments of IGF-I by acetyl-L-carnitine [39]; because this hormone could regulate the immune response, inhibiting the cellular apoptosis and modulating the immune aggression [40].

Conclusions

This paper described the use of acetyl-L-carnitine plus nicotinamide to prevent type 1 diabetes and, for the first time, the level of insulin secretion was recovered along with metabolic parameters. Throughout the studies, a number of conclusions has been reached.

Firstly, the proposed treatment was nontoxic and well tolerated. Secondly, the children at diabetic risk were followed along the study by the increments of insulin and the recovery of abnormal parameters. It is worth mentioning that no child became diabetic and this evolution required a prolonged time of treatment to be successful. Finally, we would like to highlight the efficiency of this type of treatment, and to note that these results must be confirmed by population studies.

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